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Reactivity of the Sulfhydryl Groups of Muscle Phosphofructokinase*

Robert G. Kemp and Paul B. Forest

ABSTRACT: Rabbit skeletal muscle phosphofructokinase contains 16.9 ± 0.3 cysteinyl residues per 90,000 g of enzyme. On the basis of their reactivity toward 5,5′-dithiobis(2-nitrobenzoic acid), these cysteinyl residues may be classified into five groups. A single exceedingly reactive SH group exhibits an apparent second-order rate constant that is 2×10^4 times higher than the rate of the reaction of denatured phosphofructokinase with the disulfide compound. The second class of SH groups consists of two cysteinyl residues that are somewhat less reactive and are protected from reaction by either adenine nucleotides or fructose 6-phosphate. The reaction of these two SH groups with 5,5′-dithiobis(2-nitrobenzoic acid) results in the loss of more than 90% of the enzyme activity. The third class consists of one SH group that reacts at about 0.4 of the rate of the second class. Approximately five SH groups become available for rapid reaction with 5,5′-dithiobis(2-nitrobenzoic acid) as the pH of the reaction medium is increased to 9. Two of these SH groups are protected from reaction with 5,5′-dithiobis(2-nitrobenzoic acid) by fructose 1,6-diphosphate.

The remaining SH groups react very slowly with 5,5′-dithiobis(2-nitrobenzoic acid) and can be made available for rapid reaction only if the tertiary structure of the enzyme is destroyed. A simplified procedure for the preparation of crystalline rabbit skeletal muscle phosphofructokinase is described.

Work in recent years had indicated that phosphofructokinase represents a sensitive control point in glycolysis (Lowry *et al.*, 1964) and that its activity is regulated by interaction with a number of metabolites (Lowry and Passonneau, 1966; Kemp and Krebs, 1967).

The successful preparation of highly purified skeletal muscle phosphofructokinase has been reported by a number of laboratories (Ling *et al.*, 1965; Uyeda and Racker, 1965; Parmeggiani *et al.*, 1966). These workers have indicated that the purified enzyme is not particularly stable but that it can be readily protected from inactivation by the presence of substrates. It was early recognized that phosphofructokinase is extremely sensitive to agents that destroy thiol groups (Engel'hardt and

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Sakov, 1943; Axelrod *et al.*, 1952). Furthermore, Engel'hardt and Sakov (1943) observed that ATP and fructose-6-P protected the enzyme against oxidation by various oxidizing agents.

In the light of these observations, it appeared of interest to investigate the reactivity of the SH groups of phosphofructokinase with DTNB,[1] to determine the influence of substrates and effectors of phosphofructokinase on this reactivity, and to examine the enzyme for the occurrence of SH groups "essential" for activity.

Materials and Methods

All nucleotides used in these studies were obtained from P-L Biochemicals. DTNB, disodium fructose-1,6-diP (Sigma Grade), and the sodium salt of β-glycero-P were obtained from Sigma Chemical Co. The fructose-6-P employed in this work was either Sigma Grade I or Calbiochem Grade B subsequently purified on Dowex 1 (formate) with a linear gradient from 0 to 2 M formic acid. Glycylglycine (Grade A) and the auxilliary enzymes used in the assay of phosphofructokinase were purchased from Calbiochem. Sodium dodecyl sulfate was purchased from Nutritional Biochemicals.

Phosphofructokinase was prepared from rabbit skeletal muscle by a simplified procedure that combined and modified steps of the published procedures of Ling *et al.* (1966) and Parmeggiani *et al.* (1966). A rabbit was killed by an overdose of Nembutal and bled by cutting blood vessels in the neck. The muscles from the hind legs and back were quickly removed and chilled in ice. The muscles were passed through a meat grinder at 4° and homogenized twice in a Waring Blendor for 30 sec each time in the presence of three volumes of 30 mM KF and 3 mM EDTA (pH 7.5). The mixture was centrifuged for 30 min at 10,000*g* and 2° in a Sorvall RC2 centrifuge. The supernatant was passed through glass wool to remove lipid material, placed in a −6° bath, and chilled to 0°. Two-tenths volume of isopropyl alcohol was added dropwise and the temperature of the mixture was allowed to decrease to −5°. The mixture was stirred at this temperature for an additional 20 min and the precipitate was collected by centrifugation for 30 min at 10,000*g*. The precipitate was dissolved in a volume of 0.1 M Tris-phosphate–0.2 mM EDTA–0.2 mM fructose-1,6-diP (pH 8) equal to $^1/_{15}$ of the volume of the original extract. This suspension was dialyzed for 2 hr against the same buffer. The dialyzed suspension was then placed in a 65° water bath and, with constant stirring, the temperature of the suspension was brought to 57° and held at 57–59° for 5 min. The suspension was chilled to 5° and centrifuged for 20 min at 15,000*g*. The supernatant solution was saved and the pellet was washed with a small volume of the previously mentioned Tris-phosphate buffer and again centrifuged. The enzyme was precipitated from the combined supernatant fractions by the addition of a solid ammonium sulfate to 0.5 saturation. The precipitate was collected by centrifugation and dissolved in a buffer consisting of 50 mM glycero-P, 2 mM EDTA, and 2 mM ATP (pH 7.2) to a final protein concentration of 25–30 mg/ml. The solution was dialyzed for 1 day against the same buffer containing ammonium sulfate at 0.32 saturation. The precipitate was removed and enzyme was crystallized from the supernatant solution by the technique described by Parmeggiani *et al.* (1966).

Specific activity of the crystalline enzyme was identical with that published previously and the yield of first crystals was 35–40 enzyme units/g of muscle as compared with published yields of 48 (Ling *et al.*, 1966) and 20 (Parmeggiani *et al.*, 1966) units per g.

Second or third crystals of phosphofructokinase were collected by centrifugation in the cold and dissolved in a buffer consisting of 25 mM glycylglycine–25 mM sodium glycero-P–1 mM EDTA (pH 7.2). The enzyme was dialyzed for 1 hr against this buffer and, to remove ATP, was passed through a 20 × 5 mm column containing a mixture of acid-washed charcoal–powdered cellulose (1:1). Protein concentration was measured at 279 mμ by employing the extinction coefficient of 10.2 for a 1% solution (Parmeggiani *et al.*, 1966).

All reactions of phosphofructokinase with DTNB were performed at 20° and the rate was followed at 412 mμ with a Gilford Model 2000 spectrophotometer. Reactions were started by the addition of DTNB. Titrations were performed in a buffer consisting of 25 mM glycylglycine, 25 mM sodium glycero-P, and 1 mM EDTA. As indicated in the results, glycine was substituted for glycylglycine in this buffer when titrations were performed at high pH. The maximum optical density recording for a solution of cysteine titrated with DTNB at eight different hydrogen ion concentrations between pH 6.0 and 10.0 varied by about 5%. The reported extinction coefficient of 13.6 (Ellman, 1959) at 412 mμ for the thionitrobenzoic acid ion of DTNB was employed for all calculations reported in this paper.

Studies on the binding of substrates and effectors to muscle phosphofructokinase have indicated a minimal binding unit of 90,000 g (Kemp and Krebs, 1967). From data obtained with the ultracentrifuge, Paetkau and Lardy (1967) have shown that the minimal molecular weight of fully active phosphofructokinase is 380,000, and these workers have observed under certain conditions a homogeneous though inactive molecular weight species of 93,000. In the light of these observations, 90,000 g was taken as the molecular weight of the protomer of phosphofructokinase and all sulfhydryl titration data will be expressed as the number titrated per protomer.

Results

Titration of Native and Denatured Phosphofructokinase with DTNB. A time course of the titration of phosphofructokinase at pH 7.0 in the presence and absence of sodium dodecyl sulfate is shown in Figure 1. In the presence of sodium dodecyl sulfate the protein was denatured, and the average value from four independent determinations for the number of titrable SH groups in the presence of sodium dodecyl sulfate was 16.9 ± 0.3

[1] Abbreviation used that is not listed in *Biochemistry 5*, 1445 (1966), is: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

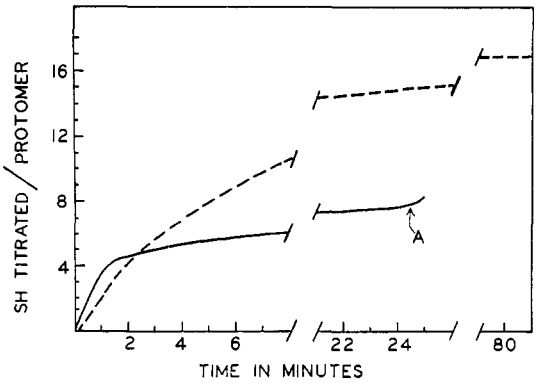

FIGURE 1: Reaction of phosphofructokinase with DTNB in the presence and absence of sodium dodecyl sulfate. Reactions carried out at 20° with a protein concentration of 0.33 mg/ml and a DTNB concentration of 0.33 mM in a buffer consisting 25 mM glycylglycine–25 mM sodium glycero-P–1 mM EDTA (pH 7.0). Solid line: no further additions. Dashed line: plus 0.3% sodium dodecyl sulfate.

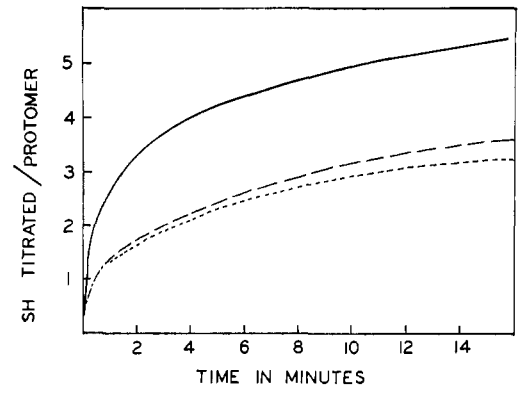

FIGURE 2: Protection of SH groups by fructose-6-P and AMP. Protein, 0.2 mg/ml; DTNB, 0.05 mM; pH 7.0; buffer described in Figure 1. Solid line: no additions; dashed line: plus 2 mM AMP; dotted line: plus 2 mM fructose-6-P.

cysteines per 90,000 g. The amino acid analysis data of Parmeggiani *et al.* (1966) indicated 15.5 cysteines/90,000 g. Hence, the presence of disulfide bonds in phosphofructokinase is unlikely.

With native enzyme, approximately four SH groups were titrated very rapidly and about seven were titrated after 25 min. At this time, indicated by point A in Figure 1, aggregation became apparent. Studies at higher pH showed that aggregation occurred only after more extensive titration of phosphofructokinase SH groups. At pH 9.5 aggregation became apparent only after the reaction of 12–13 SH groups/protomer.

With identical protein and DTNB concentrations in the two cuvets, four SH groups titrated more rapidly in the native conformation than in the denatured state. Further investigation of this phenomenon will be described later.

Protection of Sulfhydryl Groups by Substrates and Effectors. Figure 2 describes the titration of phosphofructokinase with DTNB in the presence and absence of fructose-6-P and AMP. Again, it can be seen that approximately four SH groups per protomer were titrated rapidly in the native enzyme. Protection of two SH groups per protomer was effected by either 2 mM AMP or 2 mM fructose-6-P. Glucose-6-P and fructose did not alter the titration kinetics. The effects of AMP and fructose-6-P were not additive; that is, no additional protection was observed when both compounds were included at 2 mM each. Protection against the titration of two cysteines per protomer was also observed in the presence of either 2 mM 3′,5′-cyclic AMP, ADP, or ATP. In contrast, the purine nucleotides IMP and GMP had no influence on the rate or extent of SH titration. Other effectors of phosphofructokinase kinetics, ammonium ion, citrate, and inorganic phosphate, similarly did not significantly alter the titration behavior of the enzyme SH groups when these compounds were present in the reaction mixture at 5 mM concentration.

pH Dependence of Titration of Sulfhydryl Groups in Denatured Phosphofructokinase. As indicated in Figure 1 several SH groups in native phosphofructokinase demonstrate high reactivity compared with the cysteines of denatured phosphofructokinase. In a recent review of the mechanism of sulfur–sulfur bond cleavage, Kice (1968) pointed out that cleavage can involve attack by a nucleophile, assistance by an electrophile, or both. Fava *et al.* (1957) indicated that the nucleophilic attack of a disulfide by a thiol in neutral or alkaline solution is first order with respect to both the disulfide and the mercaptide ion of the thiol. If the reactive species in the reaction between phosphofructokinase and DTNB is the mercaptide ion of cysteine, the high reactivity of the SH groups of the enzyme is the result of the reactive cysteinyl residues possessing a low apparent pK. To test the proposal that the mercaptide ion is the reactive species, phosphofructokinase denatured with sodium dodecyl sulfate was titrated with DTNB at varying pH and the data were plotted as a second-order reaction.[2] A good fit for second-order kinetics was obtained for the titration of 17 thiol groups at each hydrogen ion concentration. If a large excess of either reagent or denatured protein was employed, the reaction was pseudo-first order with respect to the limiting reactant. A plot of the log of the apparent second-order rate constant *vs.* pH is shown in Figure 3. If the reactive species is the mercaptide ion, then the observed second-order constant should vary according to the relationship, $K_2(\text{obsd}) = K_2(K/[K + (H^+)])$, where K = the dissociation constant for the protein SH group. Assuming a pK of 10.3 for the protein SH groups[3] and employing the experimental data at pH 7.0 as a reference point, the lower line indicated in Figure 3 represents the theoretical variation in the observed second-order rate constant with pH. Curvature of the theoretical line above pH 10 is apparent as the K term in the denominator of the above relationship becomes more significant. The ex-

[2] Second-order rate constant measured by determining the slope of the line in a plot of $1/(A - B) \ln B(A - X)/A(B - X)$ *vs.* time, where A = total SH content of added phosphofructokinase and B = amount of DTNB added. In kinetic plots of both native and denatured enzyme the A value was calculated on the basis of 16.9 SH groups/protomer of phosphofructokinase.

[3] Value of 10.3 is based on the pK of thioglycolic acid (Benesch and Benesch, 1955).

TABLE I: Rate Constants for Reaction of Phosphofructokinase with DTNB.[a]

Additions	No. of SH Titrated	K_2'(obsd) ($\times 10^2$ M^{-1} min^{-1})	K_2(obsd) ($\times 10^2$ M^{-1} min^{-1})
None	1.1	[c]	[c]
	1.8	20	190
	1.1	5.6	86
	(12.9)[b]	2.6	3.4
1 mM fructose-6-P	1.1	[c]	[c]
	1.1	4.7	72
	(12.8)[b]	2.2	2.9
0.3% sodium dodecyl sulfate	16.9[d]	4[d]	4[d]

[a] Data taken from Figure 4. Number titrated was determined from ordinate intercepts as discussed in the text. K_2'(obsd) was calculated from the slopes obtained from the data shown in Figure 4. These rate constants were therefore determined on the basis of 16.9 thiol groups/protomer. The number of thiol groups reacting at the indicated rate is, of course, actually that shown in the first column of numbers. The true observed second-order rate constant (K_2-(obsd)) is obtained by multiplying K_2'(obsd) by 16.9 divided by the number titrated at the indicated rate. [b] This value determined by subtracting the number of fast-reacting sulfhydryl groups from 16.9. This number cannot be determined directly due to aggregation when seven to eight SH groups are titrated. [c] Rate of titration of the most reactive SH group at pH 7.0 was too rapid to permit calculation of the rate constant. Calculation of the rate based on the earliest point detected in the titration would indicate a rate constant greater than 10^5 M^{-1} min^{-1} for this SH group. [d] These data were obtained in an independent experiment.

cellent fit of the experimental data with the theoretical line supports the proposal that the rate of the interchange reaction is dependent upon the concentration of the mercaptide ion of the protein SH groups. The observed rate constant obtained at pH 10.3 was approximately 7×10^5 M^{-1} min^{-1}. If the assumption of a pK of 10.3 for the protein SH is correct, then the actual second-order rate constant for the reaction is 1.4×10^6 M^{-1} min^{-1}. Although the experimental data display some curvature at high pH, deviation from the theoretical curve is apparent. This would suggest that the pK of the protein thiols may be slightly higher than 10.3.

Kinetics of the Titration of Phosphofructokinase with DTNB. A second-order plot of the DTNB reaction with phosphofructokinase in the presence and absence of fructose-6-P is described in Figure 4. It is apparent the titration kinetics represent a composite of the titration

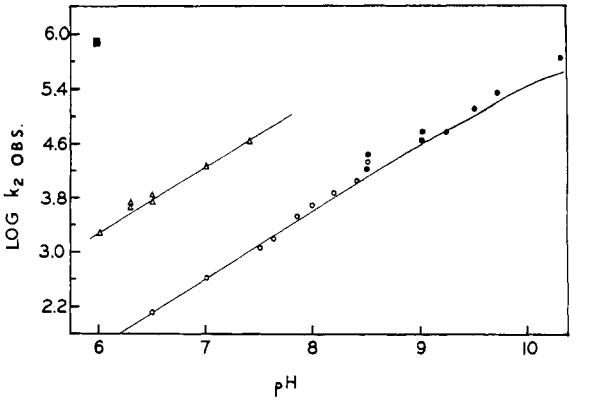

FIGURE 3: pH dependence of the rate constant for the reaction of DTNB with phosphofructokinase. Apparent second-order rate constants were determined for the following: (○) phosphofructokinase denatured with 0.3% sodium dodecyl sulfate, glyclyglycine–glycerol-P–EDTA buffer was employed; (●) denatured phosphofructokinase in the presence of glycine–glycero-P–EDTA buffer; (△) rate constants for titration of those SH groups of native enzyme that are protected by fructose-6-P (see Results); (■) rate constant for the titration of the single most reactive SH group of native phosphofructokinase. The lower line represents the theoretical variation in the observed second-order rate with pH if a pK of 10.3 for the protein SH groups is assumed (see Results).

of different classes of SH groups with varying reactivities. The intercept at the ordinate can be converted into an actual number of SH groups reacting at the indicated rate by solving for X in the relationship intercept $= 1/(A - B) \ln B(A - X)/A(B - X)$ (see footnote 2).

Table I presents both the observed second-order rate constant and number of SH groups titrated at the given rate for the data of Figure 4. In the absence of fructose-6-P three classes of SH groups displayed reactivity greater than that expected from that of the reaction of DTNB with denatured phosphofructokinase. One class of two fast-reacting cysteines was completely blocked in the presence of fructose-6-P. The observed rate constant for these two SH groups was determined from

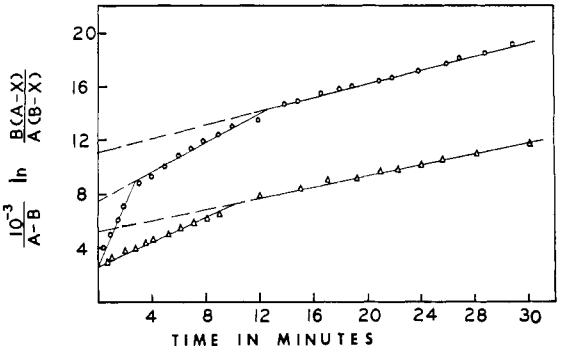

FIGURE 4: Second-order plot of SH titration of phosphofructokinase in the presence and absence of fructose-6-P. Both reactions performed at pH 7.0 in the buffer described in Figure 1. Points indicated on the graph were calculated from corresponding time points of the reaction of the enzyme with DTNB. Extrapolation of lines to the ordinate permit calculation of the number of SH groups reacting at the indicated rate. (○) No additions; (△) plus 1 mM fructose-6-P. Both reactions contained 0.2 mg/ml of enzyme and 0.03 mM DTNB.

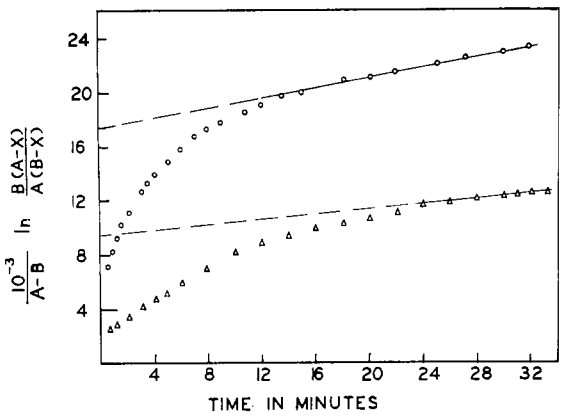

FIGURE 5: Effect of pH on the titration of SH groups in phosphofructokinase. For both reactions: protein, 0.2 mg/ml; DTNB, 0.033 mM; temperature, 20°; buffer described in Figure 1. (△) pH 6.31, additional time points were obtained to 50 min in this experiment. The points fell on a line with a slope as indicated in this figure. (○) pH 7.72.

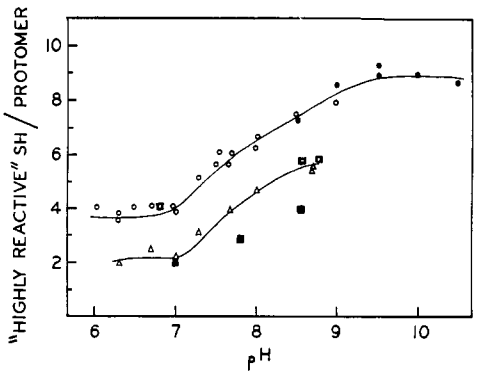

FIGURE 6: Effect of pH and fructose-1,6-diP on the number SH groups that react readily with DTNB. Number of SH groups were obtained from the ordinate intercepts as described in the Results. (○) Glycylglycine–glycerol-P–EDTA buffer was employed; (●) glycine–glycerol-P–EDTA was employed; (△) plus 2 mM fructose-6-P; (□) plus 2 mM fructose-1,6-diP; (■) plus both fructose-6-P and fructose-1,6-diP at 2 mM each.

second-order plots in the pH range where the titration was relatively slow. The rate increased approximately 20-fold from pH 6.0 to 7.4. A plot of the data as the log of the observed second-order rate constant *vs.* pH (Figure 3) gave a straight line with a slope of one. This is again consistent with the proposal that mercaptide ion is the reactive species in the disulfide-interchange reaction. If one assumes that the true rate constant for the reaction of DTNB with this SH group is identical with that obtained in the titration of denatured phosphofructokinase, then the p*K* of these two SH groups has shifted about 1.7 pH units lower than the pK of SH groups in the denatured protein.

The lowest intercept of both plots in Figure 4 indicates the presence of a single exceedingly reactive SH group per protomer. Calculations of this intercept in five different experiments gave a range of 1.0–1.2 highly reactive SH groups/protomer. To measure the rate constant for this highly reactive SH group it was necessary to study the reaction at pH 6.0 and in the presence of low concentrations of both reactants. Under these conditions, the rate of reaction of the remaining protein SH groups was relatively slow. Two independent measurements of the rate of reaction of this single SH group, yielded an observed second-order rate constant of approximately 9×10^5 M^{-1} min^{-1} (Figure 3). This rate constant is more than 20,000 times greater than the observed second-order rate constant determined in the titration of denatured phosphofructokinase at pH 6.0.

The influence of pH on the reactivity of phosphofructokinase SH groups with DTNB is indicated in Figure 5. Two effects may be observed. First, as expected, the rate of the reaction was highly dependent upon pH as indicated by the faster initial reaction at pH 7.7 than at pH 6.3. Another point of interest is the observation that the number of fast-reacting SH groups, as calculated from the ordinate intercept, increased from 3.9/protomer at pH 6.3 to 6.0/protomer at pH 7.7. Figure 6 is a plot of the number of fast-reacting SH groups at different hydrogen ion concentrations. In the absence of fructose-6-P it was observed that the number of fast-reacting SH groups remained at four between pH 6 and 7. This number gradually increased between pH 7 and 9 to reach a maximum of nine fast-reacting cysteines per protomer. These observations suggest a pH-dependent conformational change that results in an opening of the protein structure at high pH to make a greater number of SH groups available for reaction with DTNB. The number of SH groups protected by fructose-6-P was constant in the range of pH 6–9.

Also shown in Figure 6 is the effect of fructose-1,6-diP on the reactivity of the SH groups. Below pH 7.0, little or no influence of fructose-1,6-diP was observed, while at higher pH, the presence of fructose-1,6-diP partially blocked the reaction. The number of SH groups blocked approached two at higher pH and it is evident that these SH groups were not identical with those blocked by fructose-6-P as indicated by the additive effects of these two metabolites. The two SH groups blocked by fructose-1,6-diP are two of the somewhat slower reacting SH groups that are exposed as the pH is increased above 7.0.

Table II describes the loss of enzymic activity that accompanies the titration of the SH groups of phosphofructokinase. When one cysteinyl residue was titrated in the presence of fructose-6-P, approximately one-fourth of the enzyme activity was destroyed. The activity decreased slowly as two additional SH groups were titrated. In contrast, titration in the absence of fructose-6-P led to a striking loss of activity when the first three SH groups were allowed to react. A small decrease in reactivity occurred upon further reaction with DTNB. Once again, protection of two SH groups by fructose-6-P was apparent. The reaction of these two SH groups with DTNB appeared to influence profoundly the enzyme activity under the conditions of the assay. The presence of AMP during the titration of phosphofructokinase by DTNB led to enzyme activity losses identical with that described for the titration in the presence of fructose-6-P.

TABLE II: Inactivation of P-Fructokinase with DTNB.[a]

Time of Incubn (min)	No Addition		Added Fructose-6-P	
	Moles of SH Titrated/9 × 10^4 g	% Act. of Control	Moles of SH Titrated/9 × 10^4 g	% Act. of Control
0.5	1.7	32	1.2	76
3	3.0	16	1.7	74
11	4.1	9	2.4	71
20	4.7	8	2.7	67
40	5.1	8	3.2	52

[a] Sulfhydryl titration contained the following at pH 7.0 and 20°: 0.42 mg/ml of phosphofructokinase, 0.03 mM DTNB, 2 mM fructose-6-P where indicated, 25 mM glycylglycine, 25 mM glycero-P, and 1 mM EDTA. Samples were removed at indicated times and diluted 1:20 in ice-cold buffer at pH 7.5 consisting of 25 mM glycylglycine, 25 mM glycero-P, 1 mM EDTA, 0.5 mM ATP, and 0.1% bovine serum albumin (Pentex). Immediately before assay the enzyme was diluted to the final desired concentration in the same buffer. Assays were performed by coupling the reaction to the oxidation of DPNH with the use of aldolase, triosephosphate isomerase, and α-glycero-P dehydrogenase. The reaction at pH 8.2 and 26° consisted of 25 mM β-glycero-P, 25 mM glycylglycine, 1 mM EDTA, 6 mM $MgCl_2$, 4 mM $(NH_4)_2SO_4$, 0.1% bovine serum albumin, 1 mM ATP, 0.6 mM fructose-6-P, auxilliary enzymes, and 0.1 mM DPNH. The activity of the control was determined after dilution of the untreated enzyme to the same final dilution as the experimental samples.

Discussion

Studies of the sulfhydryl groups of a number of proteins have indicated that not all cysteinyl residues are equally available for reaction with a variety of reagents. Indeed, it is often the case that some of SH groups of a protein become available for reaction only after extensive denaturation. Phosphofructokinase behaves similarly in its reaction with DTNB. Depending upon the pH of the reaction mixture, four to nine of the seventeen SH groups of the protein react readily with this reagent. What is of greater interest is that within this group of reactive cysteinyl residues there are distinctive classes based upon their reactivity in the titration by DTNB. These classes may be described briefly as follows: class I, a single SH group per 90,000 molecular weight unit that is exceedingly reactive and the reactivity of which is not influenced by phosphofructokinase substrates or effectors; class II, two cysteinyl residues that are protected by fructose-6-P or adenine nucleotides and that are essential for full enzyme activity; class III, one SH group that is less reactive than classes I or II; class IV, approximately five SH groups that become available for reaction with DTNB only as the pH is increased above pH 7.0. Within class IV are two cysteinyl residues whose reactions with DTNB are blocked in the presence of fructose-1,6-diP. The number of SH groups in these classes has been related to a proposed protomer molecular weight of 90,000 g. This molecular weight, as stated earlier, is based on two observations: the binding unit molecular weight for fructose-6-P, AMP, and ADP (Kemp and Krebs, 1967); and on the subunit molecular weight of 93,000 described by Paetkau and Lardy (1967). Younathan *et al.* (1968) have recently reported that phosphofructokinase can be further dissociated into subunits of 23,000 and that peptide mapping indicated more than one kind of subunit is present. The detection of a unique SH group (class I) per 90,000 lends support to the proposal that this molecular weight unit represents the protomer weight of phosphofructokinase and suggests that at least one of the 23,000 molecular weight subunits differs from the other three.

The high reactivity in the disulfide interchange of several of the cysteinyl residues of phosphofructokinase represents an interesting problem. Sulfhydryl groups have been shown to act as acyl acceptors in proteolytic action of papain and ficin. For these enzymes it has been proposed that the SH is activated by participation in an internal thiol ester or a type of activated "high-energy" hydrogen bond (Smith and Kimmel, 1960). The most reactive SH group of phosphofructokinase would certainly fall in the category of an activated SH group. It reacts with DTNB 2×10^4 times more rapidly in the native conformation than in the denatured protein. Classes II and III are less reactive but are 50 and 20 times, respectively, more reactive in the native protein. In view of the pH dependence of the apparent second-order rate constant for the reaction of DTNB with class II cysteines and with the cysteine of denatured phosphofructokinase (Figure 3), it is tempting to attribute the high reactivities of classes I and II to a low pK for these SH groups. Employing the data of Figure 3 and the assumed pK of 10.3 for the cysteines of denatured phosphofructokinase, one may calculate a pK of 8.6 for class II cysteines and a pK of less than 6 for class I. A possible explanation for the unusually low pK for this group is that a nucleophile in the region of the SH group pulls the proton away from the sulfur atom. This would, in effect, make the cysteinyl residue a better

nucleophile for the attack at the disulfide bond of DTNB. It might be argued that the reaction of the class I cysteine, in contrast to the SH groups of denatured enzyme, proceeds by a mechanism other than nucleophilic attack at the disulfide of DTNB. Concomitant electrophilic and nucleophilic catalysis of sulfur–sulfur cleavage has also been proposed for a number of reactions (Kice, 1968). A neighboring electrophilic group on the protein could be envisioned as assisting the disulfide interchange of DTNB and the protein SH. This mechanism, however, requires a very specific interaction of the protein with the reagent. It has been noted that the class I SH group of phosphofructokinase is highly reactive in other reactions where electrophilic assistance is much less likely. The class I SH group is very readily arylated by 2,4-dinitrofluorobenzene at pH 6.8 (G. R. Putz, M. A. Potts, and R. G. Kemp, unpublished results). Under these conditions, arylation of class II and class III SH groups proceeds more slowly while the remaining SH groups of native protein and all of the SH groups of denatured protein are not arylated even after prolonged incubation with the reagent. Thus the reactivity of the class I SH group in the disulfide interchange is likely due to the increased nucleophilicity of the attacking sulfur and not to the concomitant interaction of an electrophile with the disulfide. The increased nucleophilicity of the SH group is possibly the result of an interaction with any of several amino acid side chains that can function in general base catalysis. The most likely candidates would be the carboxylate group of aspartic or glutamic acids and the imidazole ring of histidine.

It was observed that both adenine nucleotides and fructose-6-P protect against the titration of class II SH groups (Figure 2). Studies of the binding of nucleotides by phosphofructokinase have shown that cyclic 3′,5′-AMP, AMP, ADP, and ATP bind at the same site on the enzyme (Kemp and Krebs, 1967). The ineffectiveness of guanosine-5′-P and inosine-5′-P in protecting two SH groups is consistent with the binding studies. The equilibrium binding studies also indicated that at saturation 1 mole each of AMP and of fructose-6-P is bound per 90,000 g of enzyme at dissimilar sites. Hence, two SH groups are protected from reaction with DTNB by the binding of a single molecule of either AMP or fructose-6-P. It is not likely that protection is the result of an actual "covering up" of the SH groups but is rather the result of a conformational change in the protein that results in the protection. A similar conformational change must then result from the binding of fructose-6-P and adenine nucleotides at dissimilar sites on the protein molecule. Equilibrium binding studies indeed indicated dissimilar binding sites for these compounds. However, the studies also indicated a positive heterotropic effect between fructose-6-P and adenine nucleotides; that is, the binding of AMP increases the affinity of the enzyme for fructose-6-P and *vice versa* (Kemp and Krebs, 1967).

In the presence of fructose-1,6-diP the protection of two SH groups that are dissimilar from those protected by fructose-6-P is observed. In view of this observation, fructose-6-P and fructose-1,6-diP would not be expected to bind at the same site on the enzyme.

Recently, several laboratories have shown that the reaction of the SH groups of skeletal muscle and heart phosphofructokinase with a variety of reagents leads to the extensive loss of activity of the enzyme (Frenkel, 1968; Froede *et al.*, 1968; Kemp and Forest, 1968; Younathan *et al.*, 1968). In the results presented here, approximately one-fourth of the activity of the enzyme is lost upon titration of the class I SH group. Reaction of both class I and class II SH groups results in the loss of more than 90% of the phosphofructokinase activity. While the class II SH groups are protected by fructose-6-P and are essential for the expression of maximum enzyme activity, it is apparent that they are not directly involved in the binding of fructose-6-P as indicated by the residual 8% activity under conditions that would have led to a complete reaction of class II SH groups. At present it is necessary to invoke conformational changes in the enzyme structure to rationalize the observed losses in enzymic activity following titration of class I and II SH groups. Detailed kinetic analysis and studies of equilibrium binding of metabolites by the modified enzyme are under investigation and will be the subject of a future publication.

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Degradation of Sphingosine, Dihydrosphingosine, and Phytosphingosine in Rats*

Yechezkel Barenholz and Shimon Gatt

ABSTRACT: Tritium-labeled sphingosine, dihydrosphingosine, and phytosphingosine were administered by intravenous injection into rats. Two main changes were observed in the intact liver. The bases were converted in a biosynthetic route into ceramide (the *N*-acyl derivatives of the bases). In a degradative pathway they were cleaved to fatty acids which could be isolated from the liver triglycerides and lecithin. Using gas–liquid partition chromatography and determining the radioactivity of the effluents, the fatty acids, formed by cleavage of the respective bases, were identified. Hexadecanoic (palmitic) acid was the main product of the degradation of both sphingosine and dihydrosphingosine; pentadecanoic acid was the main product of phytosphingosine. The possible mechanisms leading to the formation of these fatty acids are discussed.

The three most abundant sphingosine bases are C_{18}-sphingosine (*trans*-D-erythro-1,3-dihydroxy-2-aminooctadec-4-ene; Figure 1A), C_{18}-dihydrosphingosine (D-erythro-1,3-dihydroxy-2-aminooctadecane; Figure 1B), and C_{18}-phytosphingosine (D-ribo-1,3,4-trihydroxy-2-aminooctadecane; Figure 1C). Sphingosine is the most abundant base of animal tissue sphingolipids, phytosphingosine is found mostly in plant sphingolipids, and dihydrosphingosine occurs in both tissues. These bases occur in nature in the phospho- and glycosphingolipids, which can be regarded as derivatives of ceramide, the N-acylated sphingosine base (reviewed in Hanahan and Brockerhoff, 1965). Enzymes which hydrolyze the sphingolipids of animal origin have been isolated (Gatt, 1963, 1966a,b, 1967; Barenholz and Gatt, 1966; Gatt and Rapport, 1965, 1966a,b; Frohwein and Gatt, 1966, 1967a,b, Leibovitz and Gatt, 1968; Heller and Shapiro, 1966; Brady *et al.*, 1965a,b, 1967; Kanfer *et al.*, 1966; Hajra *et al.*, 1966; Sandhoff and Jatzkewitz, 1967; Schneider and Kennedy, 1967). These enzymes can account for the complete, stepwise hydrolysis of the sphingolipids to a long-chain base, fatty acid, and the individual carbohydrate residues, or phosphorylchloline (summarized in Gatt, 1968). The metabolic fate of the sphingosine bases thus formed has been only slightly investigated. Kanfer and Gal (1966) have administered sphingosine to rats and have found that it was incorporated into ceramides and sphingomyelin. Kanfer and Richards (1967), using a similar procedure, have observed several, yet unidentified, degradation products of this base. Barenholz and Gatt (1967) have reported that tritium-labeled phytosphingosine, given by intravenous route, was converted, in the intact liver, into fatty acids, which can be completely oxidized to CO_2 and water. These fatty acids were isolated from the liver triglycerides and lecithin and identified by gas–liquid partition chromatography; most of the radioactivity was present in pentadecanoic acid. Roitman *et al.* (1967) have administered phytosphingosine and dihydrosphingosine, by intracerebral injection to rats. Similar to the results in liver, phytosphingosine was degraded in brain to pentadecanoic acid. Dihydrosphingosine, on the other hand, was degraded mostly to hexadecanoic (palmitic) acid.

This paper further extends these findings and provides evidence for the degradation of sphingosine, dihydrosphingosine, and phytosphingosine bases to fatty acids and most probably to, as yet unidentified, two- or three-carbon residues. The fatty acids can be incorporated into the liver lipids or can be completely oxidized to CO_2 and water by the fatty acid oxidation routes. These observations, together with previous data on the enzymatic hydrolysis of the sphingolipids, propose a metabolic pathway leading to the complete degradation of the sphingolipids of animal origin to CO_2 and water.

Experimental Procedure

Substrates. BIOSYNTHETIC TRITIUM-LABELED PHYTOSPHINGOSINE. *Hansenula ciferri* (strain NRRL Y-1031 F-60-10, a generous gift of Dr. Wickerham) was grown according to Maister *et al.* (1962) in a medium contain-

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